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doi:10.1016/j.ultrasmedbio.2005.09.014

● *Original Contribution*

THERAPEUTIC ULTRASOUND ENHANCES MEDIAL COLLATERAL LIGAMENT REPAIR IN RATS

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(Received 12 May 2005, revised 13 September 2005, in final form 22 September 2005)

Abstract—The purpose of this study was to evaluate the effects of therapeutic ultrasound (US) on medial collateral ligament healing. A total of 36 3-month-old male Sprague–Dawley rats with transected medial collateral ligaments were studied. Subjects were given 5-min pulsed US therapy (duty cycle; one application in 4 ms; 1:4) daily with different durations (1 day, 5 days and 10 days) and intensities (0, 0.5 and 2.3 W/cm²). After each treatment, the level of transforming growth factor beta-1 (TGF-β₁) of the ligament was measured. TGF-β₁ was not detected in the 1-day group. In the 5-day and 10-day groups, the levels of TGF-β₁ were significantly up-regulated in the high-dose subgroup ($p < 0.05$). The 10-day group also registered a significantly higher expression of TGF-β₁ than did the 5-day group ($p < 0.05$). The present findings suggest that pulsed US therapy may enhance ligament repair by up-regulating the extent of TGF-β₁ in a high-dose application. Long-term treatment with this therapy could obtain further improvement. (E-mail: rsmcpleung@polyu.edu.hk) © 2006 World Federation for Ultrasound in Medicine & Biology.

Key Words: Therapeutic ultrasound, Ligament, TGF-β₁, Healing.

INTRODUCTION

The therapeutic effects of ultrasound (US) on soft tissue injuries, such as on ligaments and tendons, had been studied extensively, but the findings were equivocal. Stevenson et al. (1986) found that US (0.75 W/cm², 3 MHz) could enhance the function of repairing tendons in chicken toes, but not the strength of the tendons. A few years later, Enwemeka (1989) studied a higher intensity of US (1 W/cm², 1 MHz) on Achilles tendon injuries in rabbits and found that both the tensile strength and energy absorption capacity of the repaired tendons were significantly improved. Takakura et al. (2002) reported that pulsed US treatment (0.03 W/cm², 1.5 MHz) could significantly improve mechanical properties such as ultimate tensile strength, stiffness and energy absorption capacity of medial collateral ligament injuries after 12-day treatment. Recently, our research team has demonstrated continuous US (1 to 2 W/cm², 1 MHz) to be effective in improving the structural properties of Achilles tendon healing in rats (Ng et al. 2003).

With increasing evidence of the therapeutic ability of US on soft tissues, researchers have tried to explore the underlying mechanism of this treatment. It was reported

that US could increase angiogenesis (Young and Dyson 1990b), fibroblast production (Young and Dyson 1990c), leucocyte adhesion (Maxwell et al. 1994), fibroblastic activities (DeDeyne and Kirsch-Volders 1995) and collagen birefringence (Cunha et al. 2001). Recently, we discovered that US could even speed up acute inflammation by up-regulating the inflammatory factors, prostaglandin E₂ and leukotriene B₄, in an intensity-dependent manner using a rat medial collateral ligament injury model (Leung et al. 2004).

Transforming growth factor-beta (TGF-β), released mainly from degranulating platelets and macrophages is one of the most important growth factors involved in tissue inflammation and repair. TGF-β has been shown to be a chemoattractant for fibroblasts (Postlethwaite et al. 1987) and macrophages (Wahl et al. 1987), to accelerate wound healing by increasing granulation tissue and re-epithelialization (Beck et al. 1991), stimulate ligament fibroblast production (DesRosiers et al. 1996), increase collagen synthesis (Deie et al. 1997) and mediate scar formation (Frank et al. 1999). In addition to its actions at the cellular level of tissue repair, Mailhot et al. (1995) also indicated that TGF-β₁ could increase the expression of proteins and the level of mRNA in human periodontal ligament *in vitro*.

The aim of the present study was to evaluate the effects of different intensities and treatment durations of pulsed (1:4) US on medial collateral ligament repair by

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measuring the expressions of the most abundant isoform, TGF- β_1 in a rat ligament injury model.

METHODS

Animals

A total of 36 3-month-old male Sprague–Dawley rats were used. The handling of the animals complied with the Code of Ethic of Animal Subjects in our university. A total of three treatment times and three US intensities were studied. Each knee of the animals was randomly allocated to a group, as shown in Table 1.

Injury protocol

The injury method replicates that of our previous study (Leung et al. 2004). In brief, the animals were anesthetized with an IP injection of a mixture of 70 mg/kg ketamine (Alfasan International, Woerden, The Netherlands) and 7 mg/kg xylazine (Alfasan). The middle part of the medial collateral ligament of both knee joints was transected. The skin wound was then closed with sutures.

Ultrasound therapy

After surgery, all the rats were given 5 min of US therapy (Enraf-Nonius, Sonopuls #434, The Netherlands) daily by means of the water-immersion method under general anesthesia. Pulsed US (duty cycle; 1 application in 4 ms; 1:4) at 3-MHz frequency was applied with different intensities: 0 W/cm², 0.5 W/cm² and 2.3 W/cm² to both legs of the animals, according to their group assignments as per Table 1. The intensities chosen covered both low-dose and high-dose low-intensity US. The animals in the 1-day group received US on postinjury day 1; the animals in the 5-day group started to receive US from postinjury day 1 to 5; and the animals in the 10-day group received US from postinjury day 1 to 10.

Sample preparation

At 1 day after the completion of US treatment, the rats were euthanized by an overdose of xylazine/ketamine injection. The ligament was harvested, weighed and immersed in 20 mM Tris-HCl in pH 7.4 with 1:2 dilution (1 g tissue: 2 mL buffer). The mixture was then homogenized. The homogenate was centrifuged at 4 °C with 12,000 rpm for 30 min. The supernatant was used for measuring the level of TGF- β_1 .

TGF- β_1 assay

TGF- β_1 was examined by the TGF- β_1 assay kit (Pro-mega TGF- β_1 E_{max}TM ImmunoAssay System, Madison, WI, USA). The supernatant was acid-pretreated and diluted by adding 4 volumes of Dulbecco phosphate-buffered saline (DPBS; 0.5 g KCl/8 g NaCl/0.2 g KH₂PO₄/1.15 g Na₂HPO₄/133 mg CaCl₂·2 H₂O/100 mg MgCl₂·6 H₂O in 1 L, pH 7.35), then acidified to approximately pH 2.6 by adding 1 μ L of 1 N HCl to each 50 μ L of diluted sample. After 15 min of incubation at room temperature, the acidified supernatant was neutralized back to pH 7.6 by adding 1 μ L of 1 N NaOH. After that, 10 μ L of TGF- β -coated mAb was added to 10 mL carbonate-coating buffer (0.025 mol/L sodium bicarbonate/0.025 mol/L sodium carbonate, pH 9.7). The plate was coated with 100 μ L of this mixture and then incubated overnight at 4 °C. After incubation, the plate was allowed to reach room temperature. The coating buffer was removed by flicking out of the wells. Then, 270 μ L of TGF- β Block 1 X buffer was added to each well at 37 °C for 35 min. After blocking, the wells were washed 3 times with Tris-buffered saline Tween-20 (TBST) washing buffer (20 mM Tris-HCl, pH 7.6/150 mM NaCl/0.05% (v/v) Tween[®]20) to remove unnecessary blocking. A total of 100 μ L of acid-pretreated sample was pipetted into each well and incubated at 37 °C for 90 min. The wells were then washed with TBST washing buffer 5 times. Then, 100 μ L of 1:1000 diluted TGF- β_1 protein antibody (pAb) was added to each well to bind the TGF- β_1 and incubated for 2 h at room temperature. After washing 5 times with TBST washing buffer, 100 μ L of 1:2000 TGF- β horseradish peroxidase conjugate was added to each well to bind to the TGF- β antibody pAb. The plate was shaken for 2 h at room temperature. The wells were then washed 5 times with TBST and 100 μ L of enzyme substrate (5 mL of 3,3', 5,5'-tetramethylbenzidine (TMB) solution/5 mL of peroxidase substrate) was pipetted into each well and the plates left to incubate for 15 min at room temperature until the solution in the wells turned blue. The reaction was stopped by the addition of 100 μ L of 1 mol/L phosphoric acid to each well. A yellow color was formed because of acidification. The absorbance at 450 nm was subsequently read by a microplate reader (Bio-tek Instrument, Inc., Winooski, VT, USA).

Table 1. The grouping of the animals

Groups	1-day Group*	5-day Group [†]	10-day Group [‡]
Control	$n = 8, 0 \text{ W/cm}^2$	$n = 8, 0 \text{ W/cm}^2$	$n = 8, 0 \text{ W/cm}^2$
Low-dose	$n = 8, 0.5 \text{ W/cm}^2$	$n = 8, 0.5 \text{ W/cm}^2$	$n = 8, 0.5 \text{ W/cm}^2$
High-dose	$n = 8, 2.3 \text{ W/cm}^2$	$n = 8, 2.3 \text{ W/cm}^2$	$n = 8, 2.3 \text{ W/cm}^2$

* 1-day treatment, euthanized at day 2; [†] 5 days treatment, euthanized at day 6; [‡] 10 days treatment, euthanized at day 11.

Statistical analysis

All values were presented as mean ± SE. Comparison of the expression of TGF-β₁ between different intensities of US was performed by one-way ANOVA. Independent *t*-test was applied in comparing the same intensity with different treatment groups. The *p* value of < 0.05 was considered to be significant. All statistical procedures were performed using SPSS version 11.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

The 1-day group

In the 1-day group, the level of TGF-β₁ in most US intensity subgroups could not be detected. This may be related to the extreme early expression of this growth factor in ligament healing. Previous studies (Martin *et al.* 1993) showed that TGF-β₁ mRNA and protein were found to be expressed only transiently after injury, from postinjury hour 1 to hour 18.

The 5-day and 10-day groups

In Fig. 1, TGF-β₁ in both the 5-day and 10-day groups was significantly higher in the high-dose subgroup than the control and low-dose subgroups (*p* < 0.05). When comparing the same intensity subgroups with different treatment days, a significant increase could

be detected in the 10-day group, especially in the high-dose subgroup (*p* < 0.05). The above findings suggest that TGF-β₁ was able to increase by high-dose pulsed US (2.3 W/cm²). Therefore, it is also surmised that a long-term and high-dose application could further up-regulate the extent of ligament TGF-β₁.

DISCUSSION

From our findings, no increase in TGF-β₁ in the 1-day group was detected. This may be caused by the expression of this growth factor in an extremely early manner, because it was reported in previous studies (Martin *et al.* 1993) that TGF-β₁ mRNA and protein were found to be expressed only transiently after injury, from postinjury hour 1 to hour 18.

In the 5-day and 10-day groups, US could significantly up-regulate the expression of TGF-β₁, especially in high-dose subgroups, but not in low-dose subgroups (Fig. 1). This positive finding illustrated the cellular contribution of US in tissue recovery apart from improving the physical properties of soft tissues (Stevenson *et al.* 1986; Enwemeka 1989; Takakura *et al.* 2002; Ng *et al.* 2003).

Soft tissue repair consists of three overlapping stages, acute inflammation, proliferation and remodeling. During inflammation, because TGF-β₁ can attract macrophages (Wahl *et al.* 1987), its up-regulation by US, as

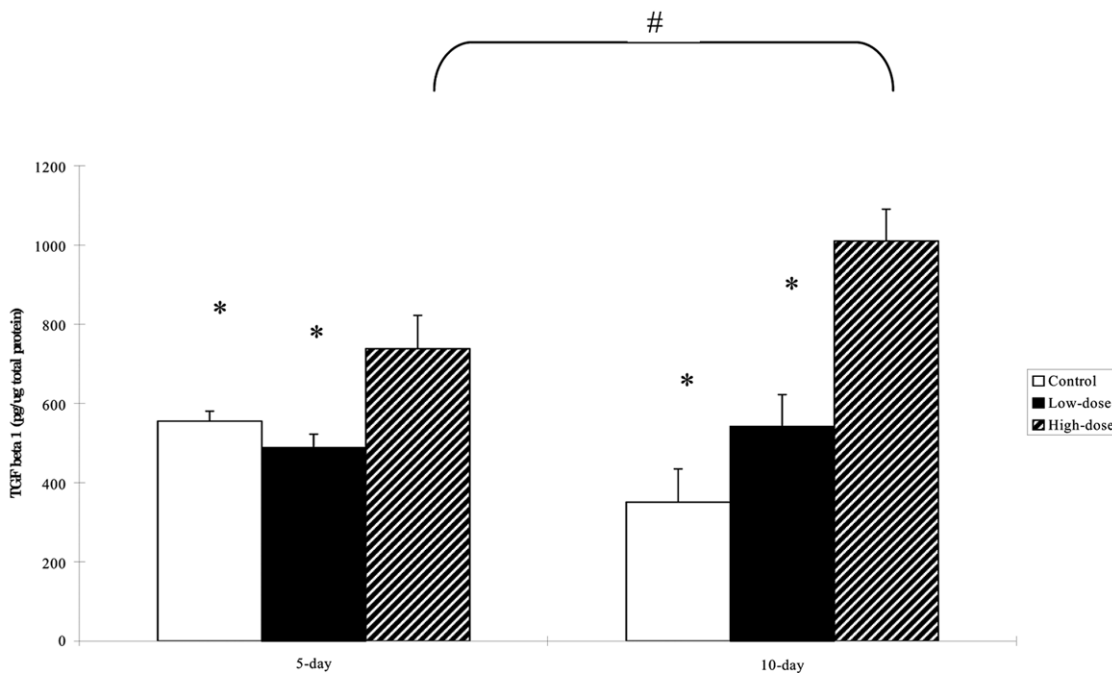


Fig. 1. Effects of US treatment of different intensities and duration on the level of TGF-β₁. Data are mean ± SE. In the 5-day and 10-day groups, TGF-β₁ was significantly increased in their respective high-dose groups. A 10-day treatment duration could further significantly up-regulate the extent of TGF-β₁ if high-dose US was applied. **p* < 0.05 compared with high-dose group; † *p* < 0.05 compared between intensity groups.

shown in the present study, could speed up acute inflammation. This interpretation was demonstrated in our previous report (Leung et al. 2004) that found US could flare up inflammation by increasing the expression of inflammatory factors, prostaglandin E₂ and leukotriene B₄.

In the proliferation stage, cells are attracted to the wound site and develop into granulation tissue. Granulation tissue is highly cellular, containing macrophages, fibroblasts, endothelial cells, fibronectin, type III collagen and hyaluronic acid. Fibroblasts are the main collagen producers for connective tissues and are responsible for wound contraction. A rise in TGF- β_1 could stimulate fibroblast chemotaxis (Postlethwaite et al. 1987), fibroblast production (DesRosiers et al. 1996) and granulation tissue proliferation (Beck et al. 1991).

In the remodeling phase, type III collagen is replaced by type I. This process continues until the collagen pattern and tissue mechanical characteristics gradually simulate the normal tissue. However, this ideal state may never be achieved in scar tissue. US could not only up-regulate collagen birefringence on rat Achilles tendons, revealing better organization and aggregation of collagen bundles (Cunha et al. 2001), but could also increase TGF- β_1 expression, as shown in the present study, which can retard the scar-formation process (Frank et al. 1999).

When comparing the same intensity subgroups with different treatment days, the most significant increase was evident in the high-dose subgroup (2.3 W/cm²). This indicates a directly proportional relationship between treatment duration/intensity and expression of TGF- β_1 as exemplified by the results obtained from the 10-day group. However, previous US studies reported that long-term application of US may not provide favorable results in tissue healing. Young and Dyson (1990a) studied two timings (5 days and 7 days) of US treatment (0.1 W/cm², 0.75 MHz and 3 MHz, 5-min daily) on the rat skin wound and reported a significant increase in the number of blood vessels only in the 5-day group, not in the 7-day group. More recently, Takakura et al. (2002) studied the effects of pulsed US (0.03 W/cm², 20-min daily) on the healing of medial collateral ligament in rats at 12- and 21-day intervals. They found that, in the 12-day treatment group, mechanical properties such as ultimate load, stiffness and energy absorption were significantly superior to those of the control group. However, the 21-day group indices were not different from those of the control group. The difference between our findings and those of the previous studies on tissue recovery may be dose-based. High-dose US may demonstrate a desirable outcome in tissue healing, as shown in the present study, particularly in long-term use.

CONCLUSION

The present findings indicate that pulsed US could enhance ligament healing by up-regulating the expression of TGF- β_1 in high-dose application. Long-term and high-dose treatment could achieve further improvement.

Acknowledgements—This work was supported by a Faculty Area of Strategic Development grant (A106) from the Department of Rehabilitation Sciences, The Hong Kong Polytechnic University. The authors thank Ms. Christine Van for editing.

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